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13. ABSTRACT (Maximum 200 Words)
Androgens are intimately associated with prostate cancer progression. We have previously identified more than 24 androgen-response genes. One of the genes encodes calreticulin, a highly conserved protein with demonstrated functions in intracellular Ca^{++} homeostasis, cell adhesion, chaperoning, and gene expression. Our studies have indicated that calreticulin overexpression is suppressive to anchorage-independent growth and metastasis of prostate cancer cells and calreticulin expression is down-regulated in human prostate tumor specimens. Thus, down-regulation of calreticulin in clinical prostate cancer specimens is likely to be an important step in prostate cancer progression. Our observations argue that part of androgen-induced gene expression program, such as calreticulin, is inactivated in the progression of prostate cancer, which represents a new concept in prostate cancer biology. Our results also provided strong basis for further exploring the mechanism by which calreticulin suppresses prostate tumor metastasis. In addition, we have generated 9 deletion mutants for calreticulin, which will allow us to determine which of the three domains, N, P, or C, is responsible for the suppression of prostate tumor metastasis.

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Introduction:

Understanding the androgen action pathway in the prostate has clinical significance.

Androgen plays an important role in the development and progression of prostate cancer (Bosland, 1992; Carter and Coffey, 1990; Kozlowski and Grayhack, 1991; Lee et al., 1995). Understanding the androgen action pathway in the prostate will provide insights into the mechanisms by which androgen impacts the pathogenesis of prostate cancer, and may lead to more effective approaches for its prevention and treatment. The androgen action pathway here is defined as a cascade of molecular and cellular events triggered by androgen manipulation leading to cell proliferation, apoptosis, and/or differentiation.

Androgen controls homeostasis of the prostate.

As shown in Table 1, androgen stimulates proliferation and differentiation in a regressed prostate but not in a fully-grown prostate. On the other hand, androgen ablation induces massive apoptosis and rapid dedifferentiation in a fully-grown prostate, but has little or no effect on a regressed prostate. These observations suggest that in the regrowth process of a regressed prostate, androgen replacement stimulates and then nullifies proliferation, establishes apoptotic potential while inhibiting apoptosis, and induces and maintains differentiation. The molecular mechanisms by which androgen controls prostate regrowth remain largely unclear.

Table 1. The impact of androgen manipulation on the regressed prostate and the normal prostate.

Androgen	Regressed Prostate	Fully-Grown Prostate
+	Proliferation & Differentiation	No Significant Change
-	No Significant Change	Apoptosis & Dedifferentiation

+ represents androgen replacement and − represents androgen ablation or administration of anti-androgens. Differentiation is defined as the expression of prostate-specific markers. Dedifferentiation is defined as loss of the expression of prostate-specific markers.

Androgen action is mediated through androgen-response genes including calreticulin.

The dramatic influence of androgen on the prostate is mediated through androgen receptor (AR). AR is a ligand-dependent transcription factor that regulates the expression of androgen-response genes, either directly or indirectly (Mainwaring, 1977; Zhou et al., 1994). Thus, androgen-response genes should mediate AR downstream events leading to cellular and morphological changes in the prostate during androgen manipulation.

To study the androgen action pathway, we have searched for androgen-response genes on the basis of their induction during the initial regrowth of the regressed ventral prostate in 7-day castrated rats using a highly sensitive PCR-based cDNA subtraction method (Wang and Brown, 1991; Wang et al., 1997). Our search has identified 25 genes that are up-regulated by androgen and 4 genes that are down-regulated by androgen in the ventral prostate of a 7-day castrated rat.

One of the androgen-response genes encodes calreticulin. Our recent studies suggest that calreticulin has significant growth suppressive role in prostate cancer and its expression is down-regulated in prostate cancer cells. These observations argue that part of the androgen action pathway, which is growth suppressive, is down-regulated in prostate cancer pathogenesis.

Calreticulin is a multi-functional Ca^{++} binding protein.

Calreticulin is an evolutionarily conserved major Ca^{++} binding protein in endoplasmic reticulum ER (Krause and Michalak, 1997; Michalak et al., 1992; Sontheimer et al., 1995). Calreticulin has been implicated in the regulation of a variety of cellular functions including the regulation of intracellular Ca^{++} homeostasis (Bastianutto et al., 1995; Liu et al., 1994; Mery et al., 1996; Zhu and Wang, 1999), cell adhesion (Coppolino et al., 1995; Dedhar, 1994; Fadel et al., 1999; Opas et al., 1996), steroid-mediated gene regulation (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), chaperone activity (Nauseef et al., 1995; Peterson et al., 1995; Vassilakos et al., 1998; Zapun et al., 1998), Zn^{++} binding, and rubella virus RNA binding. Calreticulin gene knockout mice are embryonic lethal because calreticulin is essential for cardiac development (Mesaeli et al., 1999).

Calreticulin consists of 400 a.a. residues after the N-terminal signal sequence is removed by posttranslational processing (Baksh and Michalak, 1996). It has a calculated MW of 46 kd and an apparent MW of 60 kd in SDS PAGE gel. There is a KDEL ER retention sequence at the C-terminal end of calreticulin. Calreticulin consists of at least 3 structural/functional domains (Fig. 1).

Signal Sequence

Binding sites
Integrin α
Steroid receptor
Zinc

Repeat A
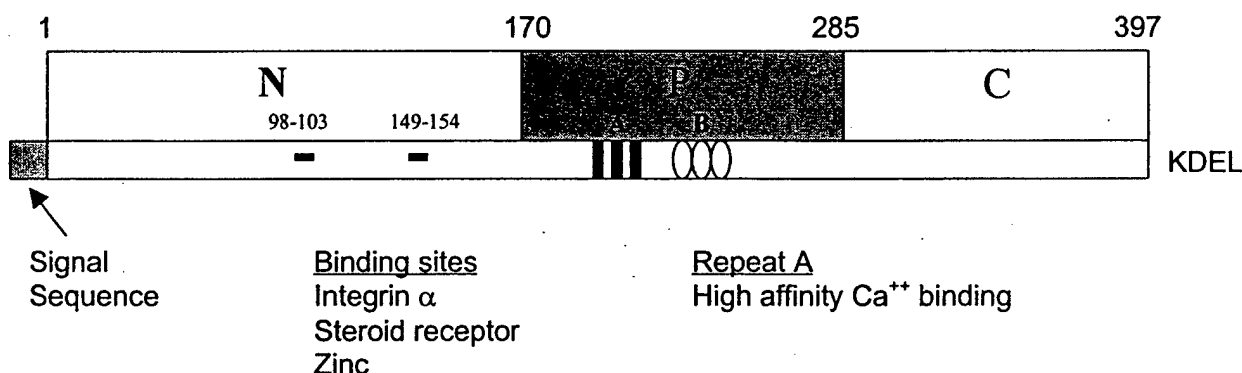High affinity Ca^{++} binding

Fig. 1. The domain structure of calreticulin (Baksh and Michalak, 1996). The N-domain (aa 1-170) of calreticulin is the most conserved domain in evolution and does not bind to Ca^{++}. The N-domain forms a globular domain with 2 regions of short α-helices at residues 98-103 and 149-154, which are responsible for the binding to integrin α and the DNA binding domain of steroid receptors. The P-domain (aa 171-285) is proline-rich and contains two sets of repeats (Repeat A and Repeat B). Repeat A consists of three repeats of aa sequence PXXIXDPDAXKPEDWDE and is believed to be responsible for high affinity (Kd = 1.6 uM) and low capacity (1 Ca^{++}/protein) Ca^{++} binding to calreticulin. Repeat B consists of three aa sequence GXWXPPXIXNPXYX and is predicted to have a rigid turn structure separating the globular head of the protein from the acidic tail. The C-domain (aa 286-397) is highly acidic and negatively charged. This large stretch of negatively charged residues binds Ca^{++} with low affinity (Kd = 0.3-0.2 mM) and high capacity (~25 Ca^{++}/protein). These high capacity low affinity Ca^{++} binding sites have led to the hypothesis that calreticulin is involved in luminal Ca^{++} storage. The C-domain in calreticulin has the most divergent aa sequence among different species.

Calreticulin is abundantly expressed and regulated by androgen in prostate epithelial cells.

We became interested in calreticulin because it was identified in our search for androgen-response genes in the rat ventral prostate (Wang et al., 1997; Zhu et al., 1998). Androgen ablation by castration rapidly down-regulates calreticulin at both mRNA and protein levels for more than 10-fold. In contrast, androgen replacement rapidly restores the expression of calreticulin in the regrowth of the castrated prostate. Northern blot analysis of tissue-

specificity of calreticulin expression showed that calreticulin expression in the prostate is much more abundant than its expression in any other surveyed organs including liver, kidney, brain, heart, muscle, and seminal vesicles. In situ hybridization and immunohistochemistry studies demonstrated that calreticulin is an intracellular protein in the epithelial cells of the prostate. Calreticulin expression in human epithelial cells is also regulated by androgens, suggesting that calreticulin regulation by androgen is conserved in evolution (Zhu et al., 1998; Zhu and Wang, 1999).

Body:

Task 1: Test the hypothesis that calreticulin down-regulation is more frequent in high Gleason grade prostate tumors (months 1-36).

a. Collect clinical specimens (months 1-36).
b. Calreticulin expression will be determined by IHC. The timing, magnitude, and frequency of calreticulin down-regulation in clinical prostate cancer specimens will be determined (months 1-36).
c. Statistical analysis will be applied to determine whether calreticulin down-regulation correlates with the Gleason grade (months 30-36).

Calreticulin expression is down-regulated in human prostate cancer specimens.

Expression of calreticulin in 21 hormone naïve clinical prostate specimens from radical prostatectomy was examined by immunohistochemistry (IHC) using an anti-calreticulin antibody (Zhu et al., 1998). These specimens contain benign regions, tumors, and/or high grade prostatic intraepithelial neoplasia (HGPIN). Calreticulin expression was down-regulated, to various extent, in 4 out of 11 HGPIN, 4 out of 10 Gleason 3 prostate tumors, and 2 out of 3 Gleason 4 prostate tumors. Examples of typical down-regulation are shown in Fig. 2. No calreticulin down-regulation was observed in benign prostatic epithelial cells in all of the specimens. These observations suggest that calreticulin down-regulation is more frequent in tumors with high Gleason score, which is associated with poor prognosis (Gleason and Mellinger, 1974). One clinically significant question is whether the cancer cells with loss of calreticulin expression will become highly metastatic and life-threatening.

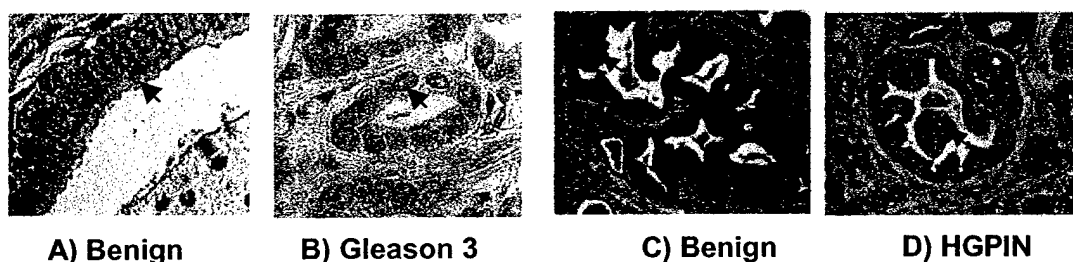| A) Benign | B) Gleason 3 | C) Benign | D) HGPIN |

Fig. 2. IHC studies of calreticulin expression in clinical prostate tumor specimens. One specimen containing both benign prostate (A) and Gleason 3 cancerous prostate (B) was stained with anti-Crt antibody and hemotoxylin as described previously (Zhu et al., 1998). Another specimen containing both benign prostate (C) and HGPIN (D) was stained with anti-Crt antibody but without hemotoxylin. Secondary antibody alone did not stain the section (Results not shown). The benign and cancerous epithelial cells are marked with arrows.

Table 1. Calreticulin immunostaining intensity in 21 human prostate tumor specimens.

Intensity	Benign (n=21)	HGPIN(n=11)	Gleason 3 (n=10)	Gleason 4 (n=3)
+	0	0	10%	33.3%
++	0	36.4%	30%	33.3%
+++	100	63.6%	60%	33.3%

The intensity of the staining was determined using specimens stained with anti-calreticulin without hemotoxylin. +++ represents normal level staining; ++ represents moderate down-regulation; + represents barely detectable expression of calreticulin.

Task 2: Determine the functional domains and motifs of calreticulin in inhibiting anchorage-independent growth (months 1-36).

a. Deletion mutants will be generated to map domain(s) essential for inhibiting anchorage-independent growth of prostate cancer cells (months 1-24).
b. Substitution mutants will be generated to map essential motifs (months 1-24).
c. The impact of calreticulin mutants on the growth of PC3 prostate cancer cells in soft agar will be tested to identify essential amino acid sequences in calreticulin (months 25-36).

Construction of mutant calreticulin expression vectors.

To prepare for mechanistic studies, we have constructed a series of mutant calreticulin expression vectors (Fig. 3). As illustrated in Fig. 1, calreticulin consists of 3 structural domains, N, P, and C. Dr. Marek Michalak has kindly provided us with HA tagged wild-type rabbit calreticulin (Rb Crt-HA) and 3 HA-tagged mutants (Rb N-HA, Rb N+P-HA, and Rb P-HA). We have made additional HA-tagged mutant calreticulin expression vectors. In addition, we have also generated GFP-tagged mutant calreticulin expression vectors, which will allow us to conveniently determine the intracellular localization of various calreticulin mutants.
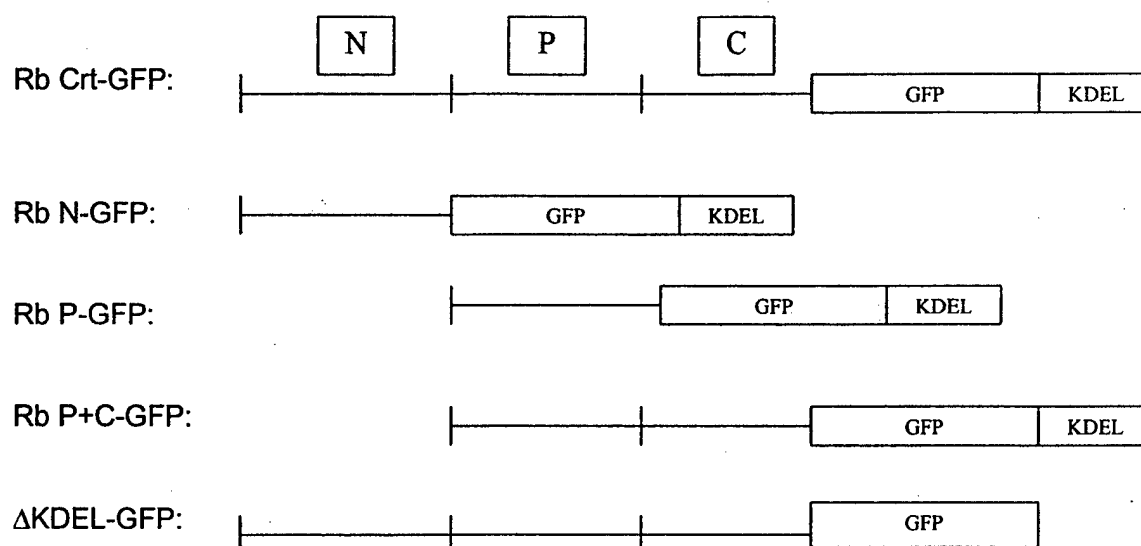
A.

7
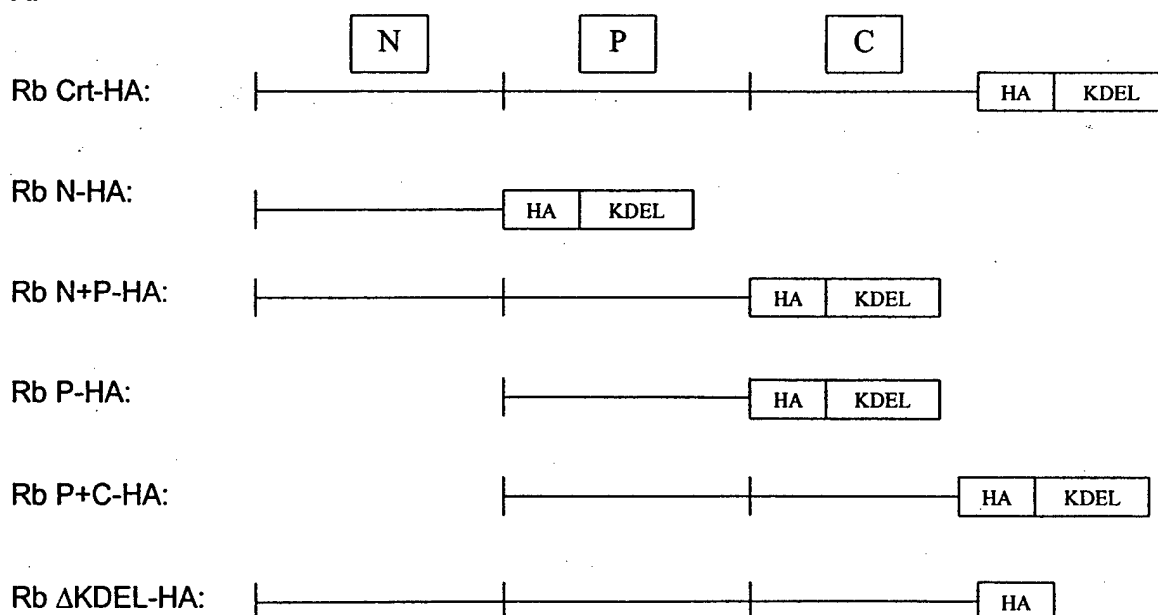
B.

Fig. 3. A. HA-tagged wild-type calreticulin and calreticulin mutants. B. Green fluorescent protein (GFP)-tagged wild-type calreticulin and calreticulin mutants. The rabbit calreticulin was used in the mutant construction. N, P, and C stand for N-domain, P-domain, and C-domain respectively (See Fig. 1 for details). All of the constructs were verified by sequencing analysis. All of the constructs have the signal peptide sequence at their N-terminus.

Task 3: Study the role of calreticulin in prostate tumor growth and metastasis *in vivo* in tumor xenografts (months 1-36).

a. The effect of wild-type calreticulin on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 32 nude mice (months 1-12).

b. The effect of calreticulin deletion mutants on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 56 nude mice (months 13-24).

c. The effect of calreticulin substitution mutants on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 56 nude mice (months 25-36).

Calreticulin markedly inhibits anchorage-independent growth of prostate cancer cells.

We cloned calreticulin cDNA into the pcDNA3.1/Hygro(+) vector (Invitrogen) to generate calreticulin (crt) expression vector pcDNA3.1/crt. The pcDNA3.1/crt was then stably transfected into PC3, a highly aggressive androgen-independent human prostate cancer cell line. Endogenous calreticulin level is very similar between PC3 clones transfected with empty vector and parental cells (Fig. 4). PC3 cells transfected with pcDNA3.1/crt express calreticulin at varying levels (2.5-10X) above the endogenous calreticulin level of the parental cell line. The highest calreticulin expression levels achieved in PC3 cells are similar to the calreticulin level in the intact rat ventral prostate (Fig. 4) and the calreticulin level in the intact rat ventral prostate represents a physiologically relevant level.
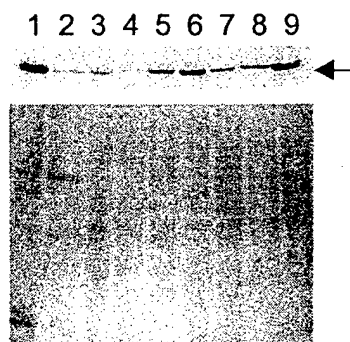
1 2 3 4 5 6 7 8 9

Fig. 4. The expression of calreticulin in parental, empty vector transfected, and pcDNA3.1/crt transfected PC3 and LNCaP cells. Lane 1 = Normal rat ventral prostate; Lane 2 = 7-day castrated rat ventral prostate; Lane 3 = Parental PC3; Lane 4 = Empty vector transfected PC3 (Mock); Lane 5 = pcDNA3.1/crt transfected PC3 clone 35 (Crt35); Lane 6 = pcDNA3.1/crt transfected PC3 clone 59 (Crt59); Lane 7 = Parental LNCaP; Lane 8 = Empty vector transfected LNCaP; and Lane 9 = pcDNA3.1/crt transfected LNCaP. The arrow indicates calreticulin. The loading of total protein was visualized by Ponceau-S staining. The Western blot represents one example of 4 experiments.
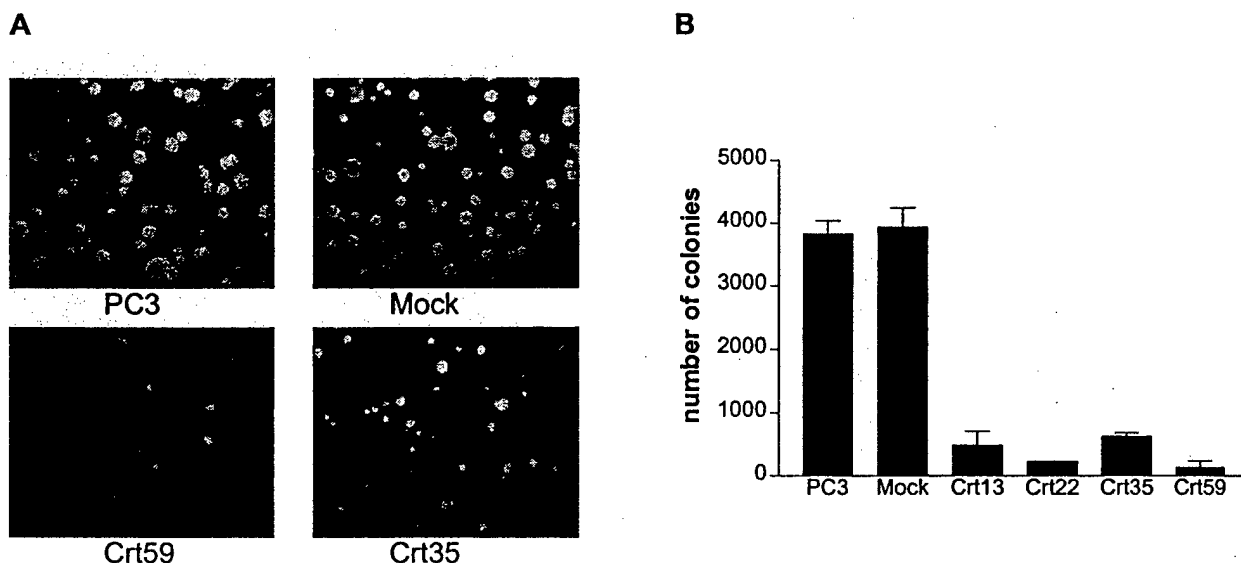
A

B

Fig. 5. The effect of calreticulin overexpression on colony formation of PC3 cells in soft agar assay. A) The colony formation of PC3, Mock, Crt 35 and Crt 59 in soft agar. The soft agar assay was conducted in 6-well plates. The bottom agar is 2 ml 0.6% noble agar containing 1X RPMI 1640 medium supplemented with10% FBS. After the preparation of bottom agar, 1 ml of 0.3 % top agar containing 5,000 cells and 1X RPMI 1640 medium supplemented with10% FBS was added to form the top layer. The assay for each PC3 subline was carried out in duplicate and was repeated at least 3 times. B) The quantification of colony formation PC3, Mock, Crt13, Crt22, Crt 35 and Crt 59. Colonies with greater than 125 um in diameter were counted in the quantification. Error bars represent standard error means (SEM).

Overexpression of calreticulin significantly inhibited the size and number of the PC3 colonies in soft agar assay (Fig. 5). Dramatic inhibition of soft agar colony formation was reproducibly observed in all four PC3 sublines with overexpressed calreticulin. Clones Crt59 and Crt35 express calreticulin at a level about 10-fold and 2.5-fold above the endogenous level respectively. Clone Crt13 and Crt22 express calreticulin about 5-fold above endogenous level. The degree of colony inhibition appears to correlates with the level of calreticulin overexpression in the four PC3 sublines. Fig. 5 shows that clone Crt59 (10X endogenous) forms less colonies in soft agar than that in clone Crt35 (2.5X endogenous). Colony formation in clone Crt13 and Crt22 (5X endogenous) is between clones Crt59 and Crt35 (Fig. 5B).

As expected, overexpression of calreticulin also markedly inhibited anchorage-independent growth of TSU prostate cancer cells (Result not shown). This suggests that calreticulin inhibition of anchorage-independent growth is a general phenomenon in prostate cancer cells. Anchorage-independent growth in soft agar correlates with the metastatic potential of cancer cells (Cifone and Fidler, 1980; Li et al., 1989). Thus, this observation suggests that calreticulin has the potential to suppress metastasis of prostate cancer cells *in vivo*.

Restoration of calreticulin expression inhibits metastasis of rat Dunning AT3.1 xenograft tumors in nude mice.

Dr. Allen Gao has generously provided us with the Dunning rat prostate cancer cell lines including the G, AT1, AT2, AT3.1, AT6.1, and Mat-Lylu, which were derived originally from the rat dorsolateral prostate. Calreticulin is down-regulated in all of the above prostate cancer cell lines (Result not shown). AT3.1 cell line was chosen as a model in this study for testing the impact of calreticulin overexpression on metastasis because subcutaneous AT3.1 xenogroft tumors readily generate countable macromatastasis in lungs of the host nude mice.

AT3.1 cells were stably transfected with the calreticulin expression vector pcDNA-Crt. Several AT3.1 sublines expressing ectopic calreticulin were established and the level of calreticulin expression in the stable lines are similar to the dorsal and lateral prostates of the rat (Data not shown). The number of lung metastases was inhibited in the nude mice bearing xenografted tumors of these sublines (Fig. 6A). In contrast, calreticulin overexpression did not inhibit the wet weight of primary tumors (Fig. 6B). These observations indicate that normal levels of calreticulin suppress metastasis rather than the growth of prostate cancer *in vivo*.
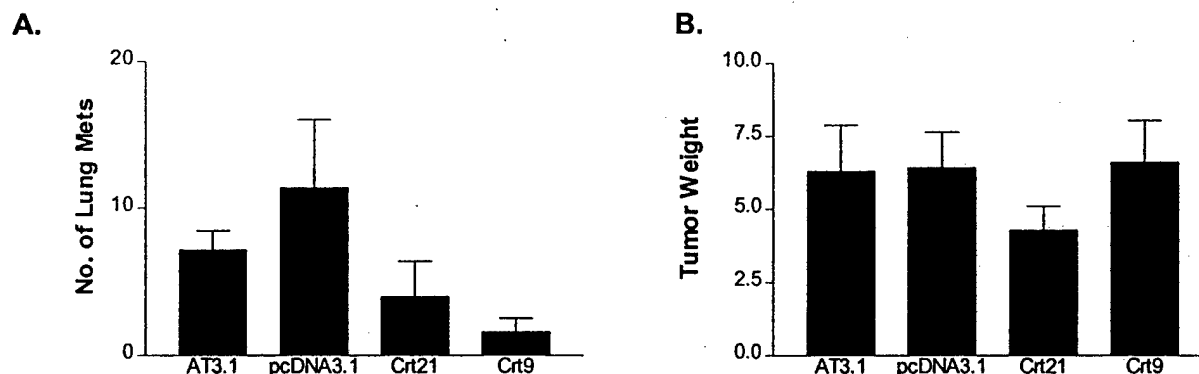

Fig. 6. A. Effect of calreticulin overexpression on AT3.1 xenograft tumor metastasis in nude mice. The parental AT3.1 (AT3.1), empty vector transfected AT3.1 (pcDNA3.1), and calreticulin expression vector transfected AT3.1 cells (Crt9, Crt21) were used to establish subcutaneous xenograft tumors in male nude mice. The cultured cancer cells were trypsinized and the single cell suspension was prepared in RPMI 1640 containing 10% FBS. Approximately 400,000 cells in 0.1 ml volume were injected subcutaneously into 4-6 week old male athymic nude mice, with 5 mice each subline (Charles River Laboratories, Frederick, MD) in the both flank. Eighteen days after injection, the tumor bearing mice were sacrificed according to a procedure approved by institutional ACUC committee. The primary tumors were excised and weighted. The lungs were fixed in Bouin's solution and lung macro-metastases were scored under a dissection microscope. B. Effect of calreticulin overexpression on the weight of AT3.1 primary xenograft tumors. The wet weight of each primary tumor was determined immediately after resection.

Key Research Accomplishments:

1. Calreticulin expression is down-regulated in human prostate cancer specimens.

Our hypothesis states that calreticulin is suppressive to prostate cancer malignancy. If calreticulin suppression of prostate cancer progression is important *in vivo*, the expression of calreticulin should be down-regulated in clinical prostate cancer specimens. Our studies provided evidence for the down-regulation of calreticulin *in vivo* in clinical specimens, which is critical to this project.

2. Calreticulin overexpression inhibits anchorage-independent growth of PC3 cells.

We have demonstrated that calreticulin overexpression markedly inhibited anchorage-independent growth of PC3 cells in soft agar assays. The levels of calreticulin overexpression correlate with the degree of the inhibition. Since the ability of cancer cells to grow in soft agar is often associated with their metastatic potential, calreticulin overexpression may suppress tumor metastasis.

3. Calreticulin suppresses metastasis in rat Dunning AT3.1 prostate tumor model.

Rat Dunning AT3.1 prostate tumor cell line is a widely used model for studying prostate tumor metastasis. Thus, this cell line was used to test the hypothesis that calreticulin overexpression is suppressive to tumor metastasis. Our studies indicate that AT3.1 cells with calreticulin overexpression generated less lung metastasis relative to the parental and empty vector-transfected AT3.1 cells.

4. Construction of calreticulin deletion mutant expression vectors.

Deletion mutagenesis is a power approach for elucidating the domain(s) that is critical for protein function(s). To identify domain(s) in calreticulin responsible for the suppression of anchorage-independent growth and/or metastasis of prostate cancer cells, we have generated 9 calreticulin mutants either tagged with HA or GFP. These mutants will provide insights into the mechanism by which calreticulin suppresses metastasis.

Reportable Outcomes:
1. We have presented an abstract entitled "Calreticulin is a potential metastasis suppressor in prostate cancer" by Mahesh Alur, Feng Jiang, Wuhan Xiao, Luping Yang, Riffat Haleem, Michael Pins, James Kozlowski, Zhou Wang to 2001 Fall SBUR (Society for Basic Urologic Research) annual meeting.

2. A manuscript entitled "Calreticulin is a potential metastasis suppressor in prostate cancer" is in preparation.

Conclusions:
Our studies have indicated that calreticulin overexpression is suppressive to anchorage-independent growth and metastasis of prostate cancer cells and calreticulin expression is down-regulated in human prostate tumor specimens. Thus, down-regulation of calreticulin in clinical prostate cancer specimens is likely to be an important step in prostate cancer progression. These observations argue that part of androgen-induced gene expression program, such as calreticulin, is inactivated in the progression of prostate cancer, which represents a new concept in prostate cancer biology. Our results also provided strong basis for further exploring the mechanism by which calreticulin suppresses prostate tumor metastasis. In addition, we have generated 9 deletion mutants for calreticulin, which will allow us to determine which of the three domains, N, P, or C, is responsible for the suppression of prostate tumor metastasis.

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Appendices: None.